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Stereochemical course of the hydrolysis reaction catalyzed by chitinases A1 and D from *Bacillus circulans* WL-12

S. Armand[a], H. Tomita[b], A. Heyraud[a], C. Gey[a], T. Watanabe[b], B. Henrissat[a,*]

[a]*Centre de Recherches sur les Macromolécules Végétales***, *CNRS, BP 53X, F-38041 Grenoble, France*
[b]*Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, 8050 Ikarashi-2, Niigata 950-21, Japan*

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Abstract

Chitinases A1 and D were purified from the periplasmic proteins produced by *Escherichia coli* HB101 harbouring recombinant plasmids carrying respectively the *chiA* and *chiD* genes of *Bacillus circulans* WL-12. HPLC analysis indicated that during the hydrolysis of chitotriose, both chitinases initially produce *N*-acetylglucosamine and only one anomer of chitobiose. ^{1}H NMR spectroscopy of the hydrolysis of chitotetraitol showed that this anomer corresponds to β-chitobiose, demonstrating that chitinases A1 and D act by a molecular mechanism that retains the anomeric configuration. This mechanism is similar to that of lysozymes although both chitinases belong to a family of proteins sharing no demonstrable amino acid sequence similarity with lysozymes.

Key words: Chitinase; Reaction mechanism; Hydrolysis; Chitooligosaccharide; *Bacillus circulans* WL-12

1. Introduction

Chitinases (EC 3.2.1.14) are glycosyl hydrolases which catalyze the degradation of chitin, a fibrous, insoluble polysaccharide made of β-1,4-*N*-acetylglucosamine residues. Chitinolytic enzymes are found in a variety of organisms, not only those where chitin is a major structural polysaccharide such as fungi [1] and insects [2], but also in bacteria and higher plants (for a review see [3]). Recently chitinases have received renewed attention since they play a role in plant defense against chitin-containing pathogens [4].

The catalytic domains of chitinases can be grouped in two families based on amino acid sequence similarities and the lack of similarity between the two families of proteins suggest that they have different folds [5]. The two families of chitinases are called families 18 and 19 in a general classification of glycosyl hydrolases [6,7]. Unlike lysozymes the mechanism of which has been extensively characterized [8,9], the molecular mechanism that prevails in each of the two chitinase families is not known. Two mechanisms have been identified for other glycosyl hydrolases: one leading to retention and the other leading to inversion of the anomeric configuration at the hydrolysis site [8]. Because the stereochemistry of the reaction product is firmly dictated by the active site structure [8], the mechanism should be conserved within a family, but not necessarily from one family to another [10]. In this study we report the first determination of the stereochemistry of hydrolysis of two recombinant chitinases belonging to family 18 of glycosyl hydrolases, namely chitinases A1 and D encoded respectively by the *chiA* and *chiD* genes of *Bacillus circulans* WL-12 [11,12].

2. Materials and methods

2.1. Purification of chitinase A1

Escherichia coli HB101 carrying recombinant plasmid pHT012 [11] was grown in L-broth medium containing 100 $\mu g \cdot ml^{-1}$ ampicillin for 18 h at 30°C. Cells were collected and periplasmic proteins were extracted by the cold osmotic shock procedure [13] using cold water containing 1 mM phenylmethylsulfonylfluoride. Ammonium sulfate was added to the extract to achieve 40% saturation. The precipitate formed was dissolved in a small volume of 20 mM sodium phosphate buffer (pH 6.0), dialyzed overnight against the same buffer and lyophilized. Chitinase A1 was purified from the lyophilisate (crude chitinase A1) by chitin column chromatography based on the method of Cabib [14] modified as follows. Crude chitinase A1 was dissolved in a small volume of 20 mM sodium phosphate buffer (pH 6.0) and applied on a chitin column (1.5 × 20 cm) previously equilibrated with the same buffer. After washing with 20 mM sodium phosphate buffer (pH 6.0) and 20 mM sodium acetate buffer (pH 5.5), chitinase A1 was eluted with 20 mM acetic acid. Elution was monitored by measuring absorbance at 280 nm and the fractions containing purified chitinase A1 were collected. The pooled fraction was adjusted to pH 6.0 with 1 N sodium hydroxide, dialyzed against 5 mM sodium phosphate buffer (pH 6.0) and lyophilized.

*Corresponding author. Fax: (33) (76) 54 72 03.

**Affiliated with the Joseph Fourier University of Grenoble

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2.2. Purification of chitinase D

Production and preparation of crude chitinase D was carried out as for chitinase A1 except that *E. coli* HB101 carrying plasmid pHTDR15 [12] was used for chitinase production and ammonium sulfate precipitation was achieved at 60% saturation. Crude chitinase D was dissolved in a small volume of 1 mM sodium phosphate buffer (pH 6.0), and applied on a hydroxyapatite column (3.9 × 6 cm) equilibrated with the same buffer. Elution was done with the same buffer and the unadsorbed protein fractions containing purified chitinase D were collected and lyophilized.

2.3. SDS-PAGE

SDS-PAGE on 10% slabs was performed as described [15] using the buffer system of Laemmli [16]. After completion of electrophoresis, renaturation of the enzymes and detection of the chitinase activity in the gel were carried out as previously reported [17].

2.4. Preparation of the chitooligosaccharides

Chitooligosaccharides were obtained by acid hydrolysis of chitin from shrimp shells. After desalting by ultrafiltration of a DIAFLO YC05 membrane, the chitooligosaccharides were separated by chromatography on a Bio-Gel P4 column eluted with water. The purity of the oligomers (chitobiose, chitotriose, chitotetraose and chitopentaose) was verified by reversed-phase HPLC using a C18 Nucleosyl column (5 μm, Interchim) equipped with a refractometric detector and also eluted with water. Chitotetraose (4.6 mg in 2 ml water) was converted into chitotetraitol by treatment with 0.5 mg sodium borohydride during 4 days. After neutralization to pH 6.0 with 50% acetic acid in water, concentration to dryness and 4 co-evaporations from methanol, chitotetraitol was purified by reversed-phase HPLC as above.

2.5. HPLC analysis of the enzymatic hydrolysis products

To determine the hydrolysis sites of chitinases A1 and D, chitooligosaccharides (DP 2-5) (200 μg) in 200 μl water were incubated at 37°C with respectively 15 μl chitinase solution (1 mg·ml^{-1} in 100 mM sodium acetate buffer, pH 5.0). After 30 min the reaction products were analyzed by reversed-phase HPLC as above. Determination of the hydrolysis products of chitinase action on chitotetraitol was performed similarly except that incubation was carried out for only 5 min on 500 μg substrate with 5 μl chitinase solution.

2.6. Proton NMR

Lyophilized chitinase A1 was dissolved in 350 μl 99.8% D_2O, lyophilized and finally resuspended in 99.95% D_2O (final enzyme concentration: 8 mg·ml^{-1}). Similarly, chitotetraitol (2.3 mg) was exchanged three times by lyophilization from 350 μl 99.8% D_2O, resuspended in 350 μl 99.8% D_2O and placed in a 5 mm NMR tube. Proton NMR was conducted at 25°C in a Bruker AC300 spectrometer equipped with a $^1H/^{13}C$ 5 mm probe and operating at 300 MHz. After recording a first spectrum of the substrate, 50 μl of chitinase solution were added to the tube which was immediately placed back in the spectrometer. The first spectrum was recorded 6 min after the addition of the enzyme. After, spectra were recorded every 5 min and a last spectrum was recorded 1.5 h after the beginning of hydrolysis.

3. Results and discussion

3.1. Purification of chitinases A1 and D

Chitinases A1 and D were obtained from ammonium sulfate precipitation of the periplasmic proteins produced by *E. coli* HB101 carrying recombinant plasmids containing respectively the *chiA* and *chiD* genes of *Bacillus circulans* WL-12. Removal of the small amount of contaminating *E. coli* periplasmic proteins and partially degraded recombinant chitinases was readily performed by chromatography on chitin for chitinase A1 and on hydroxyapatite for chitinase D. SDS-PAGE of the resulting proteins, purified chitinases A1 and D, showed

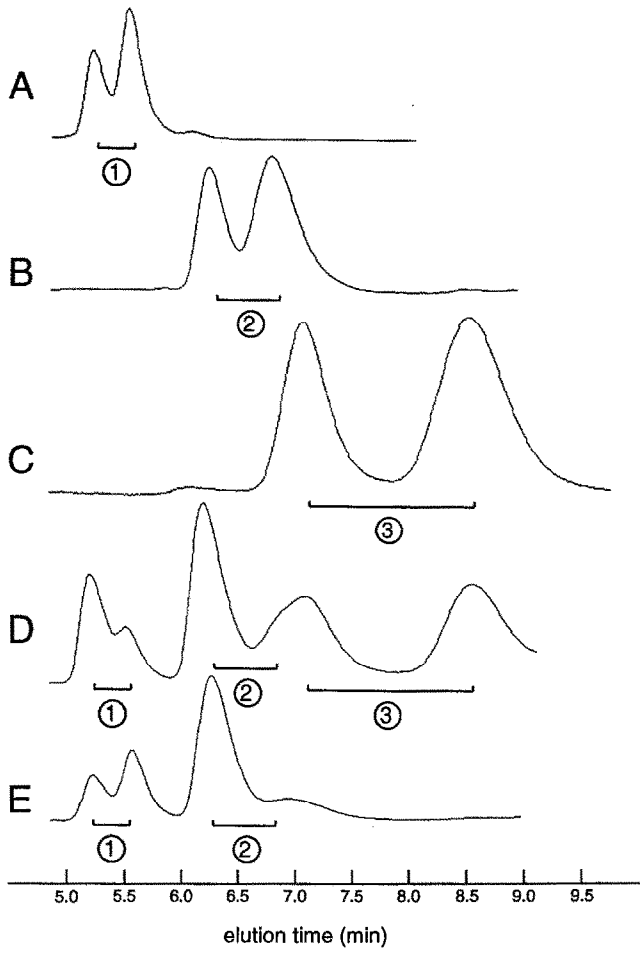

Fig. 1. HPLC analysis of chitooligosaccharides. Control: *N*-acetylglucosamine (A), chitobiose (B), chitotriose (C); the two anomers of each oligosaccharide are clearly separated (brackets labelled 1, 2 and 3 for A, B and C, respectively). Hydrolysis of chitotriose by chitinase A1 (D) and D (E): one of the two anomers of chitobiose is predominantly produced.

that they were homogenous both in protein and in chitinase activity (data not shown).

3.2. Substrate specificity of chitinases A1 and D and HPLC results

Previously, 4-methylumbelliferyl-*N,N'*-diacetylchitobioside (4-MU-GlcNAc$_2$) and 4-methylumbelliferyl-*N,N',N''*-triacetylchitotrioside (4-MU-GlcNAc$_3$) were used as substrates for chitinase A1 [11,18]. However, control experiments (not shown) have shown that the hydrolysis rate of 4-MU-GlcNAc$_2$ by both chitinases and the solubility of 4-MU-GlcNAc$_3$ were not adapted to the ^1H NMR determination of the stereochemical course of the reaction. More reactive and more soluble substrates were thus searched for among chitooligosaccharides (chitobiose, chitotriose, chitotetraose and chitopentaose) produced by acid hydrolysis of chitin. HPLC analysis showed two peaks for each pure chitooligosaccharide, indicating (i) that the two anomeric forms were separated

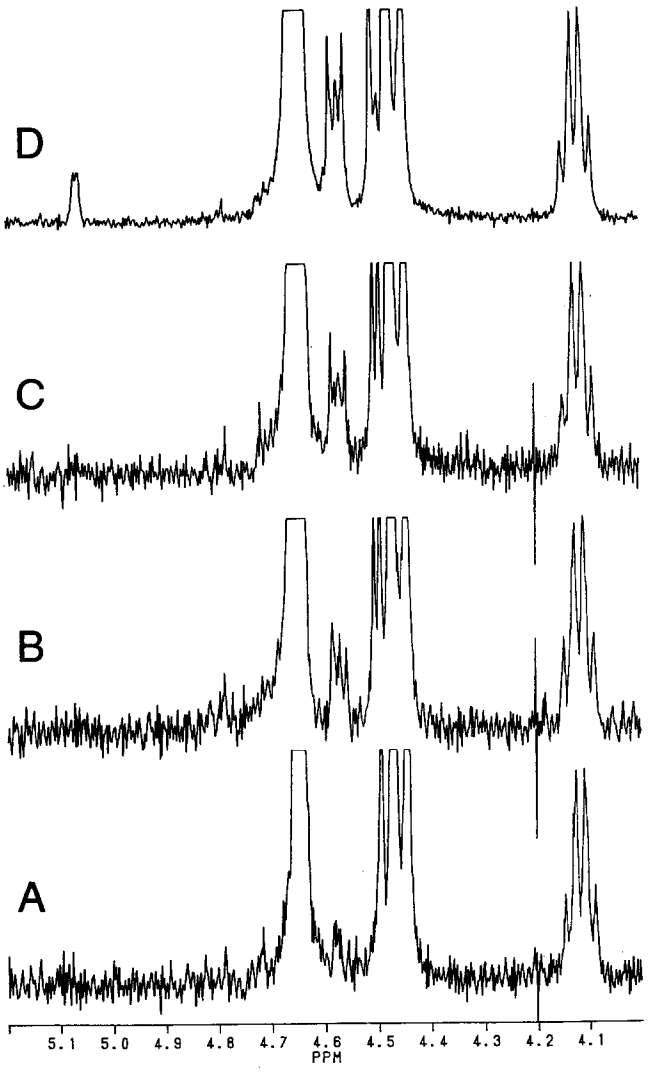

Fig. 2. ^{1}H NMR spectra of chitotetraitol during the hydrolysis by chitinase A1 in the region of the free hemiacetal H-1 resonance. (A), Reference spectrum of chitotetraitol; (B–D), same as (A) but 26, 31 and 91 min after the addition of chitinase A1.

and (ii) that mutarotation is slow in this series of oligosaccharides (Fig. 1). Chitotriose, chitotetraose and chitopentaose were readily hydrolyzed by both chitinases. The elution profile of the hydrolysis products of chitotriose was particularly interesting since it showed that the two enzymes produce *N*-acetylglucosamine and only one anomer of chitobiose (Fig. 1). In the absence of standards of α- and β-chitobiose, we could not determine which peak corresponded to which anomer, but this experiment demonstrated that the two enzymes produced the same anomer. Only one of the two chitinases was thus used for the ^{1}H NMR experiment.

3.3. NMR stereochemical analysis

For the NMR experiment, chitotetraitol (derived from chitotetraose by borohydride reduction) was choosen because it has the advantage of lacking a free reducing end which could mask that produced by the enzymatic hydrolysis. Fig. 2A shows the partial ^{1}H NMR spectrum of the starting chitotetraitol in the 4.1–5.2 ppm region. No resonance attributable to a free hemiacetal H-1 is observed, indicating that the substrate was free from contamination by unreacted chitotetraose. After 26 min of incubation of the substrate with chitinase A1, a doublet appeared at 4.57 ppm. This chemical shift corresponds to that of a free hemiacetal H-1 and its coupling constant ($J_{1/2}$ ~ 10 Hz), indicative of an axial-axial relationship between H-1 and H-2, allows the assignment of this signal to the β-anomer of chitobiose. At the same time no signal corresponding to H-1 of α-chitobiose could be detected (Fig. 2B). This demonstrates that the enzymatic reaction proceeds with retention of the anomeric configuration. The same conclusion is valid for chitinase D since it was shown by HPLC to produce the same anomer as chitinase A1 (Fig. 1). The spectrum recorded 31 min after the addition of chitinase A1 (Fig. 2C) shows that the signal β-chitobiose H-1 has grown whereas the signal of the other anomer is still not detectable, confirming that mutarotation is slow under the experimental conditions used. Fig. 2D shows the NMR spectrum 1.5 h after the addition of chitinase A1 and where the resonance of α-chitobiose H-1 at 5.1 ppm ($J_{1/2}$ ~ 3 Hz, corresponding to an axial-equatorial relationship between H-1 and H-2) is now present beside that of β-chitobiose.

4. Conclusion

Enzymatic hydrolysis of chitotetraitol by chitinases A1 and D from *Bacillus circulans* produces only β-chitobiose and chitobiitol demonstrating that these enzymes proceed with overall retention of the anomeric configuration at the cleavage site. These results represent the first non-ambiguous determination of the stereochemistry of hydrolysis of glycosidic bonds by chitinases belonging to family 18 of glycosyl hydrolases. They suggest that enzymes from this family operate by a double displacement mechanism similar to that of lysozymes [8,9]. Such a mechanism usually involves two catalytic residues promoting (i) the protonation of the glycosidic oxygen and (ii) a nucleophilic assistance leading to a glycosyl-enzyme intermediate or to the stabilisation of the transient oxocarbonium ion [8]. Glu and/or Asp residues are frequently found as the residues responsible for this type of catalysis. Only two such residues are invariant in this family of chitinases and correspond to Glu-204 and Asp-200 in chitinase A1. Site-directed mutagenesis at these residues has shown that they were directly involved in the catalytic mechanism [18].

There are analogies between chitinases and cellulases: both act on fibrous, insoluble, β-1,4-linked polysaccharides. The stereochemistry of hydrolysis has been determined for 10 out of the 11 identified cellulase families.

Five cellulase families hydrolyse the β-glucosidic bond with overall retention of the anomeric configuration while the other mechanism prevails in the 5 other families [19] and references therein). So far, only the retaining mechanism has been found in the several families of glycosyl hydrolases (chitinases and lysozymes) that hydrolyse *N*-acetyl-β-glucosaminidic linkages and for which the mechanism is known. This might only be a coincidence, although the influence of the *N*-acetyl group neighbouring the anomeric carbon cannot be excluded.

Determination of the stereochemical outcome of the hydrolysis reaction by chitinolytic enzymes from the other large family of chitinases (glycosyl hydrolase family 19) which contains only enzymes from plant origin is now necessary. Now that the two HPLC peaks corresponding to chitobiose have been unambiguously assigned to the α- and β-anomers, determination of the stereochemistry of hydrolysis chitooligosaccharides by any chitinase of any mechanism could be done using the HPLC approach described here.

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